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DIETARY FACTORS STIMULATING OOGENESIS IN *AEDES AEGYPTI*

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Vertebrate blood provides hematophagous insects with a unique oogenic stimulus, a relationship that has long intrigued students of mosquito biology but has not been fully explained. One unresloved issue concerns the central question of the nature of the oogenic stimulus. A classical study ascribed this stimulus to the physical stretching of the midgut resulting from engorgement on vertebrate blood (Larsen and Bodenstein, 1959). However, a more recent report presents contradictory evidence and suggests that only the nutrient content of the blood-meal is crucial (Bellamy and Bracken, 1971). If this were true it would require the mosquito to assess the potential nutrient available in its midgut even before apparent digestion had begun, and this information would have to be transmitted to the brain within a few minutes of feeding (Clements, 1956). No mechanism for such a rapid assessment seems evident.

Accordingly, we re-examined this basic problem. The objective of the present study was to compare the roles of various physical and chemical properties of the blood meal in stimulating *Aedes aegypti* to commence vitellogenesis.

Materials and Methods

Mosquitoes were obtained from a colony of *Aedes aegypti* isolated on Grand Bahama Island in 1972 and maintained at 24–26° C, 70% R.H. and with 16 hours of light per day. Larvae were reared on Purina guinea pig chow and pharate adults separated as to sex. Virgin, female mosquitoes were provided raisins as food and used in the experiments at 3–5 days after adult ecydsis. One day prior to the experiment, food and water were removed.

In experiments requiring measurement of the quantity of blood ingested, non-anesthetized mosquitoes were transferred to a tared vial via an aspirator and weighed on a Sartorius semi-micro balance accurate to 0.01 mg. After weighing, mosquitoes were permitted to feed on a human host until suitably engorged. Immediately following feeding, each mosquito was re-weighed and transferred to individual holding chambers.

Various solutions were introduced into the mid-guts of mosquitoes in two ways: injection *via* the anus, and artificial feeding.

Injection via *the anus*

Non-anesthetized mosquitoes were transferred to an immobilization chamber by means of an aspirator (Fig. 1). The outer (sleeve) portion of the chamber consisted of a plastic tube (2 cm long and 1 cm diameter) that was closed at one end

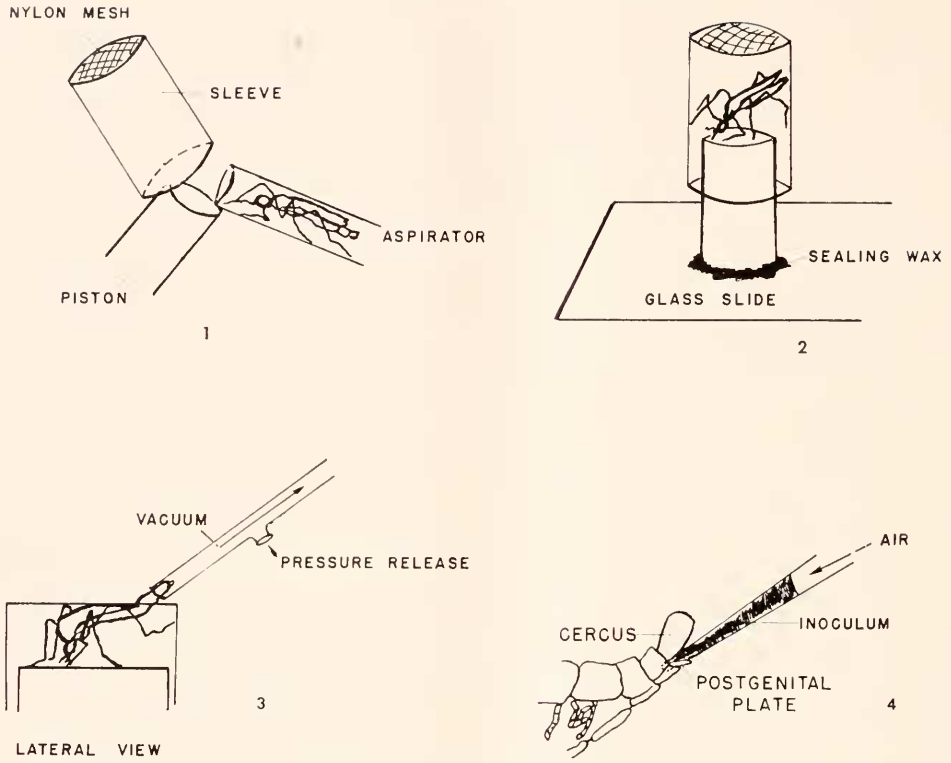

FIGURE 1. Insertion of mosquito into sleeve portion of restraining device.
FIGURE 2. Assembly of restraining device.
FIGURE 3. Method of drawing abdomen of mosquito through mesh and extension of postgenital region.
FIGURE 4. Method for anal injection. Note that malpighian tubules are visible beneath distended pleural membrane.

by a nylon mesh (tulle). The sleeve was placed over a snug-fitting post so that a mosquito confined in the sleeve would be pressed against the mesh (Fig. 2). Suction was then applied to the mosquito in order to draw the abdomen through an opening in the mesh (Fig. 3). This resulted in eversion of the terminal abdominal segments and exposure of the anus. A finely drawn pipette was then inserted superficially into the hind gut (free-hand) and fluid expelled from the pipette by means of compressed air (Fig. 4). Resulting distension of the abdomen made it possible to visualize the malpighian tubules which became pressed against the abdominal wall. Occasionally, the gut ruptured and the hemocoele rather than the midgut, filled with inoculum. When this happened, the malpighian tubules floated freely and such mosquitoes were discarded.

Artificial feeding

Solutions were placed in 2 ml watch glasses and covered with baudrouche membrane. The watch glasses were then warmed to 37° C and mosquitoes, confined

above the membrane, were permitted to feed to repletion. The ATP (0.01 M) was added as a feeding stimulant to those solutions not containing red blood cells.

Unless otherwise indicated, serum was prepared as follows: Horse serum, obtained locally, was lyophilized and re-dissolved to a desired concentration in distilled water. Resulting solutions were dialyzed for 24 hours against 0.85% NaCl (buffered to pH 7.1 with phosphate).

After feeding or injection, mosquitoes were held above water-soaked paper in individual, guaze-covered vials for two days. The ovaries of each were then removed, disrupted with a vibrating needle and examined at 430× with transmitted illumination.

The following commercial materials were employed: albumin (crystalline, bovine), albumin (5 × crystallized, egg), and adenosine triphosphate (crystalline, disodium salt) from Nutritional Biochemicals (Cleveland, Ohio); hemoglobin

TABLE I

Stimulation of oogenesis in female A. aegypti *after ingestion of various amounts of human blood*

Mg. blood ingested	No. ♀♀	% with developing oocytes
0.1–0.4	16	0
0.5–0.9	49	13
1.0–1.4	42	57
1.5–1.9	21	81
2.0–3.0	17	100

(2 × crystalline, bovine) and glutathion (reduced) from Sigma (St. Louis, Missouri); and globulin (human, Cohn Fraction IV) from Schwartz-Mann (Orangeburg, New York).

RESULTS

Partial feeding

In the first experiment, pre-weighed mosquitoes were permitted to feed individually on a human host and feeding was interrupted before engorgement was complete. The weight of blood imbibed was recorded immediately upon removal from the host, and mosquitoes were sacrificed two days later. Ovaries were removed, the follicles separated, and degree of development determined microscopically. The weight of non-blood–fed mosquitoes varied between 1.9 and 3.3 mg, being influenced by the quantity of fluid in the abdomen. Weight of the blood-meal varied between 0.1 and 4.0 mg.

Oogenesis was not initiated when mosquitoes imbibed less than 0.5 mg of blood, while all mosquitoes taking 2.0 mg or more had well-developed oocytes (Table I). Ovarian development was stimulated in about half of the mosquitoes that imbibed 1.0 to 1.5 mg of blood. Of those mosquitoes that failed to commence oogenesis, none had more than a few degenerate primary follicles, nor did secondary follicles develop when primary follicles were not stimulated.

Supplementation of partial feeding by injection

In order to determine whether distension of the midgut is prerequisite to oogenesis, saline was injected *via* the anus of partially-fed mosquitoes and ovarian development recorded after two days. Mosquitoes were permitted to feed on a human host but were removed as soon as blood could be clearly seen through the abdominal pleura. Such mosquitoes generally contained betweeen 0.5 and 1.0 mg of blood. Immediately upon removal from the host, saline (0.85% NaCl) was injected via the anus until the abdomen appeared to be fully distended. When injection was successful, blood and saline became thoroughly mixed and the total weight of blood plus saline was about 4.0 mg. During uninterrupted blood-feeding, mosquitoes normally imbibed about 2.6 mg of blood.

Of 235 partially-fed mosquitoes, 141 received saline injected *via* the anus (Table II). Almost half of these had activated primary ovarian follicles, including

TABLE II

Stimulation of oogenesis in female A. aegypti *after ingestion of trace amounts of blood (0.5–1.0 mg) and supplementation with saline or air injected* via *the anus*

Mg. blood ingested	Material injected *via* anus	No. ♀♀	% females with stimulated oocytes	
			Developing	Degenerating
0.5–1.0	—	112	12	0
0.5–1.0	Saline	141	38	9
0	Saline	27	0	0
0.5–1.0	Air	9	11	0
0	Air	9	0	0

13 in which most primary follicles degenerated. In contrast, of those that did not receive supplemental fluid *via* the anus, less than 12% had developing primary follicles and none of the remainder had more than a few degenerating follicles. When saline was administered *via* the anus of non-blood-fed mosquitoes, ovaries remained undeveloped.

Air was injected *via* the anus of other partially blood-fed-mosquitoes in order to distend the midgut without diluting blood already present there. However, this treatment appeared not to affect the developmental state of the ovary (Table II). Nor did injection of air stimulate oogenesis in non-fed mosquitoes.

Retention of injected solutions

We noted that the midgut contents of partially-blood-fed mosquitoes and of non-blood-fed mosquitoes receiving saline or air *via* the anus were generally expelled during the day following feeding or injection. This early evacuation of the gut rarely occurred following normal blood-feeding. Accordingly, we studied the relationship between serum concentration and retention of mid-gut contents. Serum was injected *via* the anus until abdomens were fully distended (about 4 mg). Unusual mortality was noted following injection of non-dialyzed, hypertonic serum.

Of 69 mosquitoes receiving twice concentrated serum, 33 died within the day following injection. In contrast, about 5% of mosquitoes died after receiving isotonic or hypotonic (diluted to twice previous volume) solutions. Accordingly, in subsequent experiments all solutions were dialyzed for 24 hours against 0.85% saline (phosphate buffered). Mortality resulting from the injection of such dialyzed, twice-concentrated solutions was about 10% while less concentrated serum produced negligible mortality. Of those mosquitoes that received serum diluted one part in ten, more than half lost the midgut contents within one day of injection (Table III). On the other hand, virtually all mosquitoes that received undiluted or twice-concentrated serum retained the inoculum.

In an attempt to prevent evacuation of the midgut, shellac was placed on the anuses of 37 saline injected and 24 air injected mosquitoes. Resulting mortality exceeded 50% during the next 2 days and the survivors were dissected at that time. Of the 12 surviving saline-injected mosquitoes, none had a distended

TABLE III

Retention of horse serum, variously diluted, during the 24 hr period after anal injection

Concentration of serum injected *via* the anus	No. ♀ ♀	% retaining inoculum
0.1×	35	43
0.25×	26	65
0.5×	168	89
1.0×	142	99
2.0×	133	98

midgut at 2 days after injection, nor were developing primary oocytes found. Instead, each mosquito had large quantities of fluid in the hemocoel and rectum. Malphigian tubules were grossly distended. Air was present in the midguts of each of the eleven surviving mosquitoes that were injected with air. It is interesting that the ovaries of six of these mosquitoes contained degenerating primary follicles. When the anus was not sealed, neither air nor saline was retained and mortality was nil; nor did the ovaries appear to be stimulated.

Effect on ovarian development of anal injection of serum

Mosquitoes were injected *via* the anus with varying concentrations and varying volumes of serum and subsequent ovarian development noted. Volume of material introduced was determined by weighing before and after injection. Mortality remained below 5% and, since 50% was the lowest serum concentration used, virtually all mosquitoes retained the inoculum. Those few that failed to do so were discarded.

Regardless of the serum preparation used, a greater proportion of mosquitoes began oogenesis when 2.0 mg or more of solution was administered as compared to 1.0 to 1.5 mg (Table IV). No progressive increase was noted at volumes above 2.0 mg. It is interesting that ovarian follicles invariably matured (once stimulated) in mosquitoes receiving 1.0 to 1.9 mg of solution, while such follicles degenerated in

TABLE IV

Stimulation of oogenesis in female A. aegypti *injected via the anus with various amounts and concentrations of serum*

Mg. serum injected	Concentration of serum injected					
	0.5×		1.0×		2.0×	
	No. ♀♀	% Stimulated	No. ♀♀	% Stimulated	No. ♀♀	% Stimulated
1.0–1.9	15	33	10	30	9	33
2.0–2.9	53	51	38	66	37	76
3.0–3.9	40	55	37	73	41	85
4.0–4.9	31	52	38	63	30	77
5.0–6.0	11	55	17	59	13	77

12% of mosquitoes receiving more inoculum. No further pattern in ovarian degeneration was evident. It is clear that the proportion of mosquitoes initiating oogenesis was correlated with the concentration of serum in the inoculum (Table IV). Differential increments between twice-diluted (0.5×), normally-concentrated (1×), and twice-concentrated (2×) serum approximated 12%.

Stimulation of ovarian development by various blood components

We then administered various components of human blood *per os* and *per anus* and compared subsequent ovarian development. Those mosquitoes that did not

TABLE V

Stimulation of oogenesis in female A. aegypti *after ingestion of solutions containing various nutrients*

Solution ingested	No. ♀♀	% females with stimulated oocytes	
		Developing	Degenerating
Fresh blood	10	100	0
Stored blood	6	67	0
Blood cells	6	67	0
Serum	4	100	0
Hemoglobin-50 mg/ml	9	0	0
Albumin 10 mg/ml	25	4	0
(Bovine)			
50 mg/ml	36	22	0
100 mg/ml	24	38	0
200 mg/ml	12	67	0
Globulin 10 mg/ml	15	38	20
25 mg/ml	14	50	7
Glutathione 0.34 mg/ml	13	0	23
3.4 mg/ml	8	13	25
Saline	15	7	0

gorge fully were discarded. Whole defibrinated blood was highly stimulatory when ingested through a membrane although prior storage seemed to reduce this property (Table V). Similarly, oogenesis was stimulated by washed cellular components and by serum alone. ATP was added to serum preparations in order to stimulate feeding.

We attempted to identify more precisely those blood components that stimulate ovarian activity. Surprisingly, ingested hemoglobin appeared not to stimulate ovarian development (Table V); on the other hand, both bovine albumin and globulin were highly stimulatory. Glutathione appeared to be slightly stimulatory. In all but one mosquito, saline was non-stimulatory. However, in that exceptional mosquito, oogenesis proceeded as after normal blood-feeding. Degeneration of primary ovarian follicles was rarely observed in this series of observations. Degeneration occurred in few (six) mosquitoes fed globulin and a similar number (five) fed glutathione.

TABLE VI

Stimulation of oogenesis in female A. aegypti *injected* via *the anus with solutions (greater than 0.002 ml) containing various nutrients (50 mg/ml)*

Solution injected	No. ♀♀	% females with stimulated oocytes	
		Developing	Degenerating
Globulin	29	41	52
Hemoglobin	29	0	31
Albumin (egg)	18	0	0

Finally, three potentially stimulatory solutions were injected via the anus and subsequent ovarian development observed. Mosquitoes were weighed both before and immediately after injection in order to insure that each received at least 2 mg of solution. All mosquitoes in this experiment retained the inoculum at one day after treatment and more than 80% survived at two days. Globulin solutions administered via the anus stimulated oogenesis in more than half of the mosquitoes treated (Table VI). Follicles degenerated in only a few (three) mosquitoes. In contrast, hemoglobin was less stimulatory; 9 of 29 treated mosquitoes had degenerating follicles and none proceeded to mature eggs. Egg albumin was apparently non-stimulatory.

DISCUSSION

Ovarian development in anautogenous mosquitoes is arrested at a specific developmental stage, a condition normally sustained until the female gorges on vertebrate blood. Our observations on *A. aegypti* confirm previous reports (Colless and Chellapah, 1960; Roy, 1936; Volozina, 1967; Woke, Ally and Rosenberger, 1956) that resumption of development requires ingestion of a certain threshold volume of blood. A similar threshold effect has been reported for *Culex pipens* (Hosoi, 1954; Kupriyanova, 1966). It is interesting that other mosquitoes may differ in this regard; female *Culex tritaeniorhynchus,* for example, produce a

few eggs when only trace amounts of blood are taken (Mogi, Wada and Omori, 1972).

These observations suggest that female *A. aegypti* may assess the quantity of blood present in the midgut by means of some volumetric measure. Since the threshold value for oogenesis appears to be about 1.5 mg of blood per mosquito and these mosquitoes normally imbibe about 2.6 mg of blood, completion of oogenesis requires that the midgut be nearly fully engorged. Indeed, when a mosquito has taken 1.5 mg of blood, it seems to be well distended and ingested blood is readily visible through the stretched pleural membrane. The oogenic signal is generated only after the mosquito is nearly completely filled.

Time relationships seem to confirm that a volumetric measure may contribute to the assessment of the nutrient content of a blood-meal. An ovary stimulating signal is generated within the head of female *A. aegypti* within 30 minutes of the completion of blood-feeding (Clements, 1956) and primary oocytes commence micro-pinocytosis and R.N.A. synthesis within an hour (Anderson and Spielman, 1971; Anderson and Spielman, 1973). At this time the mass of blood in the midgut appears to be virtually undigested indicating that an exclusively chemical-nutritional assessment is unlikely. Solely on the basis of exclusion, reception of a physical stimulus seems to be required.

Since the oogenic signal is generated after injection of nutrient through the anus, any physical estimate of food volume must involve sensations of degree of distension or of pressure within the abdomen. An estimate based on the quantity of flow through the anterior gut would be excluded. Larsen and Bodenstein (1959) have suggested that the oogenic signal is based on an assessment of the degree of distension of the midgut.

Our observations provide experimental confirmation that the volume of nutrient present in the midgut is crucial to the release of the oogenic stimulus. Eggs develop after saline supplementation of blood meals that would otherwise be too small to stimulate oogenesis. It is paradoxical that supplementation by anal injection of air fails to enhance the oogenic stimulus since, in contrast to injection of saline, injected air does not dilute the blood meal.

In addition to demonstrating the importance of physically derived oogenic stimuli, our observations confirm Bellamy and Bracken's (1971) suggestion that female mosquitoes can assess the chemical nature of their midgut contents. Working with *Culex pipiens*, these investigators found that eggs mature following repeated daily hemocoelic injection of a concentrated mixture of amino acids. Although this suggests that products of protein digestion, themselves, might transmit an oogenic signal from the midgut via the hemolymph, such non-physiologic manipulation does not constitute rigorous proof. One such digestion product, isoleucine, deserves special attention. This amino acid appears to be an essential dietary component required by *A. aegypti* for the production of eggs (Greenberg, 1951). Our observations confirm that proteins such as hemoglobulin which are poor in isoleucine generally fail to stimulate oogenesis.

Since oogenesis requires a combination of stimuli that are quite specific for vertebrate blood we might speculate that the role of the ventral diverticulum may not be to prevent stimulation of oogenesis by a sugar meal (Larsen and Bodenstein, 1959). When sugar solutions are delivered to the midgut, even in great volume,

the ovaries are not affected. Indeed, unless this food happens to be isotonic with its body fluids, the insect will rapidly die. One role of the diverticulum would be to protect the midgut epithelium from osmotic stress. The wall of this inert diverticulum is highly impermeable to water (Clay and Venard, 1972) and contained fluids are only slowly released to the midgut where digestion and absorption take place.

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Summary

1. Female *Aedes aegypti* generally fail to commence oogensis unless they imbibe veterbrate blood nearly to repletion. However, oogenesis frequently proceeds if a partial blood meal is supplemented with injection of saline via the anus. Injection of saline or air alone generally does not stimulate the ovaries.

2. When the midgut is fully distended with serum the proportion of mosquitoes developing eggs is correlated with concentration of serum. Similarly, feeding on the cellular fractions of blood, on globulin and albumin fractions of serum, and to a lesser extent on glutathione, stimulates oogenesis. Hemoglobin, administered either per os or per anus is relatively non-stimulatory and egg albumin appears to be without oogenic effect.

3. These observations suggest that oogenesis depends upon distention of the midgut as well as on the presence of sufficient concentrations of specific chemical moieties.

4. A principle function of the ventral diverticulum may be to protect the midgut against osmotic stress rather than to prevent premature oogenesis.

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